The role of the microRNA *mir-125a-5p* in mouse skeletal development

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Abstract

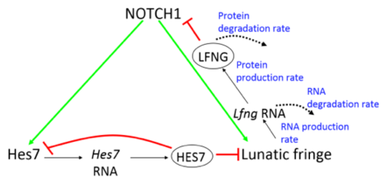
In all vertebrates, the ribs, vertebrae, and repeating dorsal muscles develop from repeating tissue blocks called somites that form early in development. The regularity of somite formation depends on a "clock" of oscillating genes, which includes Lunatic fringe (Lfng). Oscillatory expression of Lfng is essential for normal somitogenesis and skeleton formation. In chick, the microRNA *mir-125a-5p* binds to and destabilizes *Lfng* mRNA, allowing rapid clearing of *Lfng* mRNA during the "off" phase of each oscillation. Loss of *mir-125a-5p* in chick results in disorganized somitogenesis. Whether *mir-125a-5p* plays a similar role in mammals is unknown. The purpose of this study is to determine if *mir-125a-5p* regulates Lfng expression during somitogenesis, and thus axial skeleton formation, in mice.

To determine the role of *mir-125a-5p* in somitogenesis, CRISPR-Cas9 targeted mutagenesis was used to create mice lacking *mir-125a-5p*. *Lfng* expression in *mir-125a-5p* mutant and wild-type mouse embryos was compared via in situ hybridization. Effects on somitogenesis were determined by performing in situ hybridization for *Uncx4.1*, a somite boundary marker, in mutant and wild-type embryos. To directly investigate the results of *mir-125a-5p* loss on skeletal development, the skeletons of older embryos were stained with Alcian Blue and Alizarin Red to mark bone and cartilage.

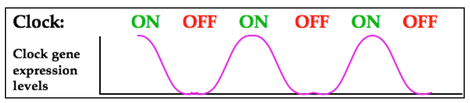
There was no significant difference in somite formation or skeletal development between mutant and wild-type embryos, indicating that *mir-125a-5p* does not have the same vital role in mammalian somitogenesis as it does in chick. It may be that *mir-125a-5p* is uninvolved in somitogenesis in mice; alternatively, a similar miRNA could compensate for *mir-125a-5p* loss. Interestingly, some adult male mice lacking functional *mir-125a-5p* appear to be less fertile and show defects in spermatogenesis; this could be due to the location of *mir-125a-5p* within an intron of one isoform of Spaca6, a gene involved in sperm-egg fusion.

Background: Lfng and the segmentation clock

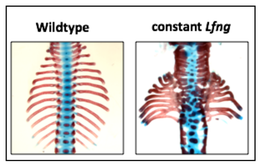
- The regular pace of somitogenesis is set by a "clock" of gene products interacting in feedback loops [2]. Some of the major clock genes are shown below.



- These interactions result in oscillatory expression of clock genes. Each cell in the pre-somitic mesoderm (PSM, the precursor to somites) experiences alternating "on" and "off" phases of clock gene expression, shown below.

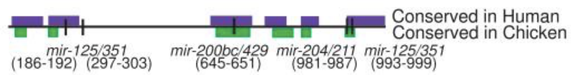


- The mRNA of one clock gene, Lunatic fringe (*Lfng*) [3], must be cleared from the cell during each oscillation's "off" phase. Overexpression of *Lfng* during this phase causes skeletal defects [4], including rib fusions and bifurcations, as shown below.

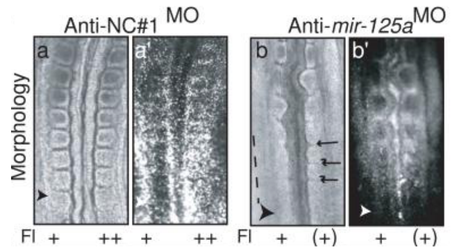


Background: *mir-125a-5p* in chick

- In chick, *mir-125a-5p* binds and destabilizes *Lfng* mRNA [5]. Its binding sites, as shown, are highly conserved.



- In chick embryos, *mir-125a-5p* knockdown leads to disorganized somitogenesis [5], as shown below.

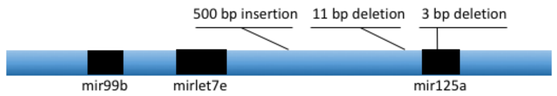


Hypothesis

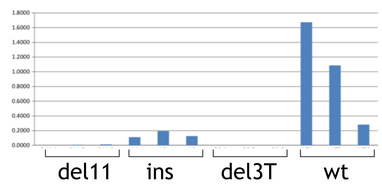
During mouse somitogenesis, *mir-125a-5p* binds to and destabilizes *Lfng* mRNA, leading to efficient downregulation of *Lfng* expression in the "off" phase of each oscillation. In mice lacking *mir-125a-5p*, *Lfng* mRNA will be degraded more slowly. This will have one of two possible consequences:
The segmentation clock will be slowed, and fewer somites will form per length of spine.
Alternatively, oscillations will break down altogether, resulting in disordered somites and misshapen vertebrae and ribs.

Results

- CRISPR-Cas9 targeted mutagenesis was used to create mice deficient in *mir-125a-5p*.
- Three mutations were created: a large insertion before the *mir-125a-5p* seed sequence (ins), a, 11 bp deletion immediately before the seed sequence (del11), and a 3bp deletion within the seed sequence (del3T). These mutations are shown below in the polycistronic cluster from which mir-125a-5p is transcribed.

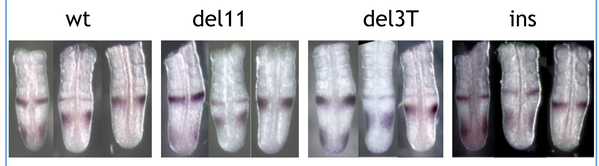


- Quantitative RT-PCR was used to evaluate *mir-125a-5p* expression in each mutant. As shown below, the del3T and del11 mutations resulted in complete loss of expression of *mir-125a-5p*, while in ins mutants expression was severely reduced. (Expression levels are shown normalized to *sno234*, a constitutively expressed snoRNA.)

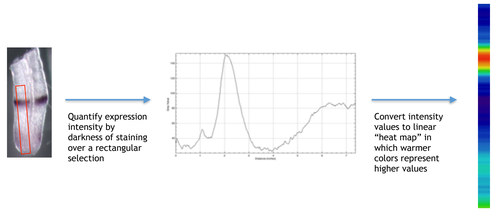


Results (contd)

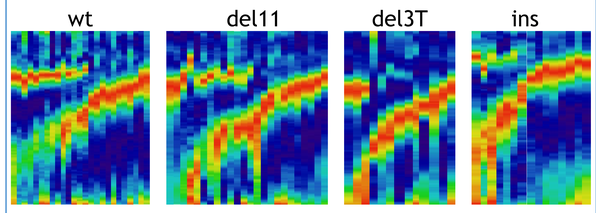
- Lfng expression of *mir-125a-5p* mutant and wild-type mice was visualized by RNA in situ hybridization for *Lfng* mRNA. Shown below are tail buds of 10.5 dpc mouse embryos; for each genotype, the 3 phases of oscillation are shown. *Lfng* mRNA is marked in purple.
- There was no obvious difference in *Lfng* expression between *mir-125a-5p* mutant and wild type embryos.



- *Lfng* expression intensity was converted into a linear "heat map".

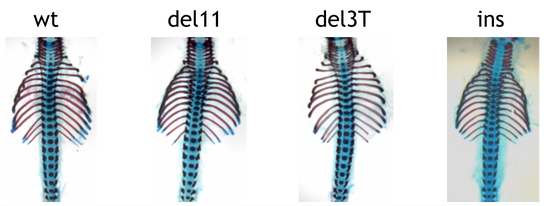


- Heat maps from many embryos in each phase of oscillation were combined to reveal complete oscillations of *Lfng* expression.
- There was little difference in *Lfng* oscillations between mutant and wild-type embryos.



- To directly examine the mutation's effect on somitogenesis, somites of mutant and wild-type 10.5 dpc embryos were marked by RNA in situ hybridization for *Uncx4.1*.
- *Uncx4.1* is expressed in the posterior compartment of each somite, and is marked with purple in the embryos below.
- Mutants did not have defects in number or patterning of somites.

- Skeletal development was investigated by staining bones and cartilage with Alizarin Red and Alcian Blue, respectively.
- Mutant skeletons displayed no defects.

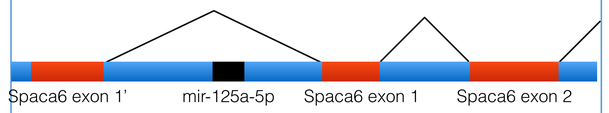


Conclusions

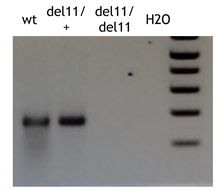
The microRNA *mir-125a-5p* is not essential in mouse somitogenesis, as it is in chick. There are several possible reasons for this difference:

- *mir-125a-5p* has no role in mouse somitogenesis
- A similar miRNA, such as *mir-125b*, is compensating for the loss
- Chick experiments knocked out *mir-125a-5p* with morpholinos, causing a sudden drop. Mouse embryos in this study lacked the miRNA from conception, perhaps allowing regulation of development.

Interestingly, del11 mutant males are infertile (n=4), and may show defects in sperm formation. This could be due to *mir-125a-5p*'s location within an intron of an alternate isoform of Spaca6, a gene participating in sperm-egg fusion [6].



RT-PCR reveals that del11/del11 males do not express *Spaca6*, as shown below. This suggests that *mir-125a-5p* production is linked to *Spaca6* splicing.



Future Directions

Further work will focus on the mechanistic reasons for the infertility phenotype. While the del11 mutation does not hit the *mir-125a-5p* seed sequence, it may lie within the binding site for Drosha, which is responsible for cleaving the miRNA hairpin out from the linear pri-miRNA. Drosha cleavage of intronic microRNAs has been shown to promote intron excision [7]. Thus, if the del11 mutation prevents Drosha from interacting with the intron, Spaca6 may be improperly spliced, leading to infertility.

References

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Acknowledgments

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